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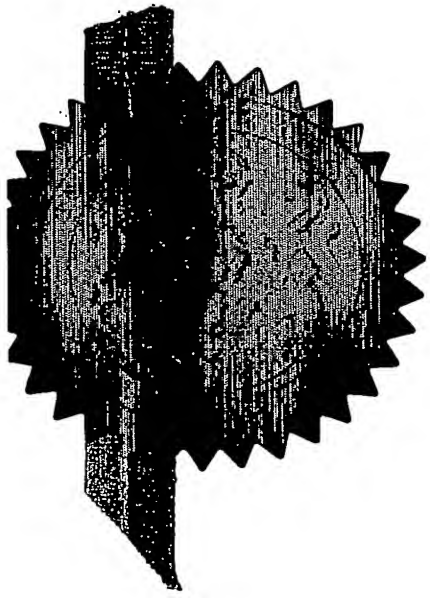

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	Patents ADP number (*if you know it*)	77366300 1
	If the applicant is a corporate body, give the country/state of its incorporation	ENGLAND.
4.	Title of the invention	NANOPARTICLE CONJUGATES AND METHOD OF PRODUCTION THEREOF
5.	Name of your agent (*if you have one*)	**W.P.THOMPSON & CO.**
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Coopers Building, Church Street, Liverpool, L1 3AB.
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DESCRIPTION

NANOPARTICLE CONJUGATES AND METHOD OF PRODUCTION THEREOF

The present invention relates to nanoparticle conjugates, in particular those which are useful in biomolecular assays, and to methods for their production.

Recent developments in nano-technology have suggested that it may be possible to use nanoparticles in detection, diagnostics, sensing and other applications (*Review Article: Angew. Chem. Int. Ed., 40 (2001) 4128-4158*). In so doing, however, it is important to remember that the quality of the nanoparticle is only one of several factors that contribute to the sensitivity and reliability of these applications. The sensitivity depends on the minimum signal that can be distinguished from the background, and any factors that contribute to this will reduce the sensitivity. In biomolecular assays a major source of background is non-specific adsorption and any conjugation method should seek to minimize this. In free solution the reaction between two binding molecules such as an antibody and its corresponding antigen is extremely fast, and a further requirement of any conjugation method is that, as far as possible, it should not hinder this reaction. One of the most exciting opportunities to have emerged from the integration of nanotechnology and analytical chemistry, is the possibility of fine tuning the optical properties of nanoparticles so that more than one analyte can be determined in the same sample (*J. Am. Chem. Soc., 123 (2001) 5164-5165*), but if reliable results are to be obtained it is important to ensure that conjugated molecules are unable to exchange from one particle to another.

Inorganic nanoparticle conjugates that interact with biological systems have recently attracted widespread interest in biology and medicine. These conjugates are believed to have potential as novel intravascular probes for both sensing (e.g., imaging) and therapeutic purposes (e.g., drug delivery) (Proc. Natl. Acad. Sci. USA, 99 (2002) 12617-12621). The requirements of these conjugates are in many respects similar to those used in biomolecular assays, but a further requirement is that the conjugate should be biocompatible and, for *in vivo* applications, should be biodegradable or able to pass through the biological particulate filter known as the reticuloendothelial system.

Most proteins contain chemical groups (-NH_2, -SH_2, etc.) that bind strongly to certain types of nanoparticle, but if necessary the number of such groups can be increased with, for example, a thiolating reagent such as 2-iminothiolane, or by genetic engineering. Fine details of the process by which proteins become non-specifically bound to nanoparticles are not well understood, but it has been postulated that a series of electrostatic and chemical interactions accompanied by conformation changes is involved. The most widespread use of this method is for conjugating antibodies to gold nanoparticles (*J. Histochem. Cytochem., 36 (1988) 401-407* and *Biotechnic & Histochem., 75 (2002) 203-242*). Excess antibodies are incubated with the nanoparticles for a short time under alkaline conditions. Then unbound protein is removed and the conjugate is stabilized with a blocking agent such as polyethylene glycol. The optimal amount of protein required for conjugation can be determined by means of a flocculation assay. When electrolytes are added to incompletely conjugated particles they flocculate. The flocculation of gold nanoparticles can be monitored by the

decrease and/or red shift of the plasmon absorption band at about 520 nm. Non-specifically conjugated proteins stabilize the nanoparticles by mutual repulsion. The minimum amount of protein needed to prevent flocculation is determined by titration and often corresponds to a single monolayer bound to the surface of the nanoparticles. Other proteins have been conjugated by similar methods, and small molecules such as oligonucleotides and haptens can be conjugated after covalent attachment to a suitable carrier protein. The main problem with this conjugation method is that bound proteins are known to desorb (*Immuno-gold Labeling In Cell Biology [Verkleij and Leunissen eds.] CRC Press, Boca Raton, Fl, (1989) pp49-60; J. Histochem. Cytochem. 39 (1991) 37-39*).

Monovalent methods of conjugation involve incubating the nanoparticles with an excess of molecules that comprise a chemical group that binds to the particles and a binding molecule that can participate in biomolecular or other applications (as shown in Figure 1). Unbound molecules are removed in a subsequent purification step such as ultra-centrifugation or gel exclusion chromatography. Monovalent conjugation may also be the first step in a more complicated protocol. A recent conference report described the conjugation of long chain mercaptoalcohols to gold nanoparticles (*Presented by V.H. Perez-Luna at the Nanotechnology in bioengineering: applications to detection, diagnostics and sensing conference, 7th Nov, 2002, Indiana, USA*). Subsequently the alcohol groups were activated with epichlorohydrin and conjugated to a layer of dextran. Carboxylic acid groups were introduced into the dextran with bromoacetic acid and then coupled to biotin. This is an adaptation of a protocol used to lay down immunosorbent surfaces on macroscopic gold substrates for surface plasmon

resonance (SPR) assays (as detailed in WO-A-90/05303), but when it is used with nanoparticles the large number of chemical and purification steps results in low yields.

Molecules conjugated to nanoparticles by functional groups such as –SH and $-NH_2$ are in equilibrium with unbound molecules in solution. They must be tightly bound to ensure that they remain conjugated, but even mercapto compounds bound to gold can dissociate (*Langmuir, 17 (2001) 4836-4843*). One solution to this problem is to conjugate molecules to the particles multivalently by more than one functional group. The equilibria that exist between bound molecules and their corresponding dissociation products are shown in Figure 2. Monovalent conjugates are characterized by a single dissociation constant K_d which depends on the affinity of individual functional groups for the nanoparticle, but multivalent conjugates are characterized by a series of dissociation constants (combined in K_t) that involve cooperative binding. There are several reports of conjugates based on divalent molecules such as dihydrolipoic acid (Figure 3) and one describing the use of trivalent aminotrithiolate (Figure 4) for conjugating antibodies to metallic nanoparticles (as detailed in U.S. Pat. No. 5,945,293). The latter compound was originally synthesized for multivalent attachment of peptides to macroscopic gold substrates (*Science, 261 (1993) 73-76*). It remained bound to these substrates even when it was heated to 180°C for 7 days.

The extent to which a functional group bound to a nanoparticle enhances the binding of a second functional group to the same nanoparticle depends on the distance by which the functional groups are separated, and the amount of steric hindrance exerted by the chemical structure that links them together. Dextrans are

flexible polymers of glucose that are known to resist non-specific adsorption and allow fast kinetics in biomolecular assays (*Sensor Actuat. B-Chem., 5 (1991) 78-84; Biomaterials, 21 (2000) 957-966*). The same flexibility that favours fast kinetics also reduces the amount of steric hindrance between functional groups in the same multivalent molecule. When primary amines are substituted into dextrans the aminodextran (amdex) products can form multivalent conjugates with metal and semiconductor nanoparticles (see U.S. Pat. Nos. 5,248,772; 5,552,086 and 5,945,293 and *Langmuir, 16 (2000) 3107-3118*). Aminodextrans have been used to conjugate biochemically active molecules such as ouabain to gold nanoparticles (*Eur. J. Cell Biol. 45 (1987) 200-208; Invest. Opthalmol. & Vis. Sci., 26 (1985) 1002-1013; J. Cell Biol., 105 (1987) 2589-2601; J. Bacteriol., 169 (1987) 3531-3538*).

The problem of nanoparticle conjugate dissociation can also be avoided by entrapping the particles in a shell from which they are unable to escape (Figure 5). Metallic and semiconductor nanoparticles can be entrapped in a shell formed by polymerization of mercaptopropyltrimethoxy silane (*Langmuir, 13 (1997) 3921-3926; Chem. Mater. 14 (2002) 2113-2119*). The shell confers stability on the nanoparticles and further reaction with other silanes introduces functional groups that can be covalently attached to binding molecules such as *biotin (Chem. Mater. 14 (2002) 2113-2119*). nanoparticles can also be entrapped by cross-linking dextran with epichlorohydrin (*Bioconjugate. Chem. 10 (1999) 186-191; Bioconjugate Chem. 11 (2000) 941-946*). Primary amines can be introduced into the dextran by reductive amination and covalently attached to proteins. Nanoparticle conjugates prepared in this way have been used for non-invasive

magnetic resonance imaging and cancer therapy. When nanoparticles are exposed to a succession of polyelectrolyte solutions of alternating charge they become entrapped in a polymer shell that is robust enough to remain intact when core particle is dissolved. Gold nanoparticles coated with an anionic layer of monovalent mercapto compounds have subsequently been entrapped in alternate layers of sodium poly(styrenesulfonate) and poly(diallyldimethylammonium) chloride (*J. Phys. Chem. B, 103 (2001) 6846-6852*). Although this layer-by-layer (LbL) approach has not yet been used to prepare nanoparticle conjugates, it has been used to conjugate antibodies to polystyrene microbeads (*J. Colloid Interface Sci., 234 (2001) 356-362*).

The equilibrium between a molecular binding site and its complex with the corresponding target molecule can be represented as follows:

$$[B] + [T] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [B:T]$$

where B and T represent the binding site and the target molecule respectively. At equilibrium the rate of complex formation is equal to the rate of complex dissociation ($k_a = k_d$) and the equilibrium constant (K_a) may be calculated from the ratio of free and complexed reactants. Applying the law of mass action:

$$K_a = \frac{[B:T]}{[B]\ [T]}$$

When the binding site is part of an antibody the equilibrium constant (K_a) is known as the affinity constant or intrinsic affinity of the antibody; this constant

is independent of the number of antibody binding sites. The antibodies most often used in immunoassays have two binding sites, which may increase the equilibrium constant by up to three orders of magnitude. This higher value is known as the functional affinity of the antibody, and it may increase even further when more than one antibody is conjugated to the same nanoparticle. Because the sensitivity of an analytical method depends to a certain extent on the affinity of a binding molecule for its target it might be thought that the aim of any conjugation method should be to maximize the functional affinity, but reported work shows that high functional affinity decreases the sensitivity in reagent limited biomolecular assays (*Anal. Chem., 74 (2002) 841-847*). When nanoparticle conjugates with a high functional affinity are used the equilibrium shown in Figure 6 prevails. 1A predominates because conjugates that bind to one separation phase analogue have a much higher probability of binding to a second analogue, than they have of binding to the analyte. High concentrations of analyte are required to shift the equilibrium in favour of 1C and sensitivity is low. When low functional affinity nanoparticle conjugates with a single binding site are used the concentrations of 2A and 2C in Figure 6 are approximately equal, because the probability of binding a solid phase analogue or the analyte is almost equal. Low concentrations of analyte shift the equilibrium to the right in favour of 2C and high sensitivity is possible.

Even when a biomolecular assay does not involve a competitive step it may still be advantageous to limit the number of binding sites conjugated to each nanoparticle. Figure 7 shows schematically what happens when nanoparticles are used to enhance the sensitivity of surface plasmon resonance immunoassays. The

number of bound nanoparticles, and hence the extent of signal enhancement, increases as the number of binding sites per nanoparticle decreases; similar considerations apply to other reagent excess formats such as sandwich immunoassays, although high functional affinity may be advantageous for some protocols (*Anal. Chem., 73 (2001) 2254-2260*). DNA arrays are widely used to compare the relative level of RNA transcripts in different cell or tissue samples (*BioEssays, 18 (1996) 427-431; BioTechniques, 33 (2002) 620-630*) (as illustrated in Figure 8). DNA corresponding to the control and experimental samples is labelled with two different organic dyes and hybridised to a single array bearing thousands of probes. The results are determined by measuring differences in fluorescence. For this to work it is essential that there is no more than one DNA molecule per fluorescent dye, and if the advantages of nanoparticles are to be harnessed for similar applications there must be only one DNA molecule per nanoparticle.

The similarity in size between nanoparticles and biological molecules makes them suitable for in vivo applications. Nanoparticle conjugates that interact with biological systems have recently attracted widespread interest in biology and medicine. These conjugates are believed to have potential as novel intra-vascular probes for both sensing (e.g., imaging) and therapeutic purposes (e.g., drug delivery). The requirements of these nanoparticle conjugates are in many respects similar to those used in biomolecular assays, except that the analytical molecules are replaced with other functional molecules. Superparamagnetic metal oxide nanoparticles entrapped in a layer of dextran and conjugated to a membrane translocating peptide can be internalised by living cells. The cells can be detected

by NMR imaging and could be retained on magnetic separation columns (Bioconjugate. Chem. 10 (1999) 186-191). More recent work has shown that semiconductor nanoparticle conjugates coated with a lung-targeting peptide accumulate in the lungs of mice, whereas two other peptides specifically direct the conjugates to blood vessels or lymphatic vessels in tumours (Proc. Natl. Acad. Sci. USA, 99 (2002) 12617-12621). These results suggest that it will be possible to prepare target selective nanoparticle conjugates for disease sensing, *in vivo* tracking and drug delivery.

Most prior art conjugation methods seek to control the number of binding molecules bound to a nanoparticle at the level of the final conjugate, when they are present at low concentrations and determination is subject to interference from the particles. This makes it necessary to use indirect methods such as electrophoresis to find out how many binding molecules are present (*Nano Lett., 1 (2001) 32-35*).

It would be advantageous to provide a nanoparticle conjugate for use in biomolecular assay, and in other applications, for which the number of functional molecules conjugated to the nanoparticles could be determined by more straightforward analytical techniques than has hitherto been the case. It would also be desirable to provide a nanoparticle conjugate which could be determined with greater sensitivity and/or reliability than has been known until now. It would be a further advantage to provide a nanoparticle conjugate which was relatively stable, preferably highly stable and not readily dissociated. It would also be advantageous to provide a method for producing a nanoparticle conjugate for use in biomolecular assays or other applications in which the numbers and types of

molecules conjugated to the particle could be more accurately controlled than has hitherto been the case. It would also be desirable too if such a method for producing the nanoparticle conjugates allowed the number and type of molecules conjugated to the particle to be determined more accurately that has been known until now.

It is an object of the present invention to provide such nanoparticle conjugates and methods for making them. One particular object of the invention is to provide nanoparticle conjugates that, when used in a biomolecular assay, are able to resist non-specific binding, will allow fast kinetics, and/or will resist displacement and exchange reactions. Another object of the invention is to provide nanoparticle conjugates with controlled functional affinity. Yet another object of the invention is to provide a nanoparticle conjugate comprising a controlled number of binding molecules, for example a single binding molecule, for biomolecular assay applications.

Accordingly the invention provides a method for the preparation of nanoparticle conjugates comprising:

a) providing a first reagent comprising a flexible hydrophilic polymer;

b) providing a second reagent comprising at least one functional molecule capable of being substituted into the flexible hydrophilic polymer;

c) providing a third reagent comprising nanoparticles;

d) contacting the first reagent with the second reagent for a period of time and under conditions effective to allow substitution of the at least one functional molecule into the flexible hydrophilic polymer;

e) before, during and/or after step d) providing the flexible hydrophilic polymer with a plurality of further substituents capable, optionally after deprotection, of binding to the nanoparticles to provide an intermediate product comprising the flexible hydrophilic polymer substituted with the at least one functional molecule and a plurality of substituents capable, optionally after deprotection, of binding to the nanoparticles;

f) if necessary, deprotecting the plurality of substituents capable of binding to the nanoparticles; and

g) contacting the, if necessary deprotected, intermediate product of step e) with the third reagent for a period of time and under conditions effective to allow binding of the, if necessary deprotected, intermediate product with the nanoparticles to provide the nanoparticle conjugates

wherein the number of functional molecules conjugated per nanoparticle in the final step is controlled by at least one of:

- controlling, by means of suitable selection of reagents and reaction conditions, the number of functional molecules substituted into the flexible hydrophilic polymer in step d);
- controlling, by means of suitable selection of reagents and reaction conditions, the number of optionally protected substituents capable of binding to the nanoparticles substituted into the flexible hydrophilic polymer in step e); and
- controlling, by means of suitable selection of reagents and reaction conditions, the number of intermediate product molecules binding to the nanoparticles in step g).

The method of the invention allows the preparation of nanoparticle conjugates wherein the number of functional molecules thereon can be controlled according to the end use of the nanoparticle conjugate. The target end product of the method of the invention is a nanoparticle conjugate having a desired number of functional molecules per nanoparticle and the method of the invention permits selection of conditions which yield the desired target end product. If the desired number of functional molecules per nanoparticle is small then the degree of control permitted by the method of the invention is close, even allowing the operator of the method to reliably ensure that, for example, only a single functional molecule is conjugated to a nanoparticle. Thus, if the nanoparticle conjugate is intended for use in biomolecular assay, the functional molecule may be an assay molecule and the number of such assay molecules per nanoparticle conjugate is controlled (probably to a relatively small number) with this end use in mind. However, if the functional molecule is intended for use in drug delivery, for example, the number of such drug delivery or drug molecules per nanoparticle conjugate is controlled (probably to a relatively large number) with this end use in mind. The degree of control under these circumstances is less close but is still significant in that the operator is able to ensure, for example, that a large number of functional molecules conjugate to the nanoparticle. The functional molecules may be directly functional (as assay molecules for example) or their functionality may be indirect in that they may be capable of binding further to other functional molecules.

Control of the number of functional molecules per nanoparticle is preferably achieved by at least one of:

- selecting the relative sizes of the flexible hydrophilic polymer ("size" in this case being a function of at least one of molecular weight, chain length and degree of chain branching) and the nanoparticle to control the number of molecules of flexible hydrophilic polymer, and therefore of optionally deprotected intermediate product, which can be accommodated on the surface of the nanoparticle;
- selecting the relative concentrations of the first and second reagents in step d) to control the number of functional molecules substituted into each molecule of flexible hydrophilic polymer; and
- selecting the number per molecule of flexible hydrophilic polymer of substituent molecules capable, optionally after deprotection, of binding to the nanoparticles.

Thus, the method of the invention may comprise determining at least approximately the desired number of, if necessary deprotected, intermediate product molecules to be bound to each nanoparticle in step g) and selecting the relative size of the flexible hydrophilic polymer and the nanoparticle such that the number of, if necessary deprotected, intermediate product molecules which can be accommodated on the surface of each nanoparticle at least approximately matches the desired number. Preferably, especially when the desired number is small (for example less than about 10, less than about 5, from about 1 to 3 or only 1), the number of, if necessary deprotected, intermediate product molecules which can be accommodated on the surface of each nanoparticle at least almost exactly matches the desired number.

The method of the invention may also comprise determining at least approximately the desired number of functional molecules to be substituted into each molecule of flexible hydrophilic polymer in step d) and selecting accordingly the reagent concentrations and reaction conditions in step d).

The method of the invention may also comprise determining at least approximately the desired number of substituent molecules capable, optionally after deprotection, of binding to the nanoparticles to be substituted to each molecule of flexible hydrophilic polymer in step e) and selecting accordingly the reagent concentrations and reaction conditions in step e).

Preferably the relative sizes of the flexible hydrophilic polymer and the nanoparticle are selected to be effective to allow binding in step g) of a controlled number of the, if necessary deprotected, intermediate product molecules with the nanoparticles.

Also provided in accordance with the invention is a method for the preparation of a nanoparticle conjugate comprising:

i. providing a first reagent comprising a flexible hydrophilic polymer having a plurality of at least one type of conjugation substituent capable, optionally after deprotection, of binding to a nanoparticle;

ii. providing a second reagent comprising at least one functional molecule suitable for binding to target molecules, optionally in a biomolecular assay, and capable of being substituted into the flexible hydrophilic polymer;

iii. providing a third reagent comprising nanoparticles capable of binding to the conjugation substituents of the flexible hydrophilic polymer;

iv. contacting the first reagent with the second reagent for a period of time and under conditions effective to allow binding of the functional molecules to the flexible hydrophilic polymer and provide an intermediate product comprising the flexible hydrophilic polymer substituted with the at least one functional molecule; and

v. contacting the intermediate product of step iv) with the third reagent for a period of time and under conditions effective to allow binding of the intermediate product with the nanoparticles to provide the nanoparticle conjugate.

Because the number of functional molecules binding to each molecule of flexible hydrophilic polymer can be closely controlled (by selecting the respective concentrations of the first and second reagents, for example), and because the number of intermediate product molecules conjugating with the nanoparticles can be closely controlled (by selecting the respective sizes of the flexible hydrophilic polymer and the nanoparticles and in some cases controlling the respective concentrations of the intermediate product and the third reagent, for example) the method of the invention allows the production of a nanoparticle conjugate comprising a nanoparticle and a flexible hydrophilic polymer bound to the nanoparticle, the flexible hydrophilic polymer (and hence the nanoparticle) being

substituted with a known number of functional molecules per nanoparticle conjugate.

Accordingly the invention provides a method for conjugating one or more functional molecules to a nanoparticle. More specifically the invention in some cases concerns a method for substituting known numbers of one or more functional molecules into a flexible hydrophilic polymer that is also substituted with chemical groups that are capable of binding to a nanoparticle, and a method for conjugating a known number of substituted polymer molecules to a nanoparticle. The invention accordingly provides a method for producing nanoparticle conjugates having a number of functional molecules that is at least approximately known even without analytical determination of the nanoparticle conjugate after it has been prepared.

The invention also provides a method for producing a nanoparticle conjugate comprising:

I. providing a nanoparticle having a surface area x;

II. providing a flexible hydrophilic polymer having a chain length and degree of branching such that a molecule of the polymer has the capacity, when suitably conformed, to envelop a surface area x/y;

III. substituting into the polymer a plurality of conjugation substituents capable of binding to the nanoparticle and at least one functional substituent capable of imparting a biomolecular or other function to the nanoparticle conjugate; and

IV. conjugating approximately y molecules of the substituted polymer to the nanoparticle via the said plurality of conjugation substituents;

wherein the flexible hydrophilic polymer is selected with regard to the variable y to provide a nanoparticle conjugate having an at least approximately predetermined number of flexible hydrophilic polymer molecules per nanoparticle. In one preferred application of this method y = x and the nanoparticle conjugate is for use in, for example, reagent limited biomolecular assays and/or DNA expression analysis. In another preferred application of this method y is considerably greater than x and the nanoparticle conjugate is for use in for example reagent excess biomolecular assays and drug delivery.

The sensitivity of nanoparticle-based biomolecular assays is partly dependent on their functional affinity (number and affinity of assay binding sites per nanoparticle) and an aspect of this invention is to provide a method for exercising control over the functional affinity of nanoparticle conjugates.

In one preferred method of the invention the plurality of substituents capable of binding to a nanoparticle comprise mercapto (-SH) groups or alternatively either disulphide (-S-S-) or thiolester (-COS-) groups that can be deprotected to provide ultimately the same conjugation as would be derived from mercapto groups. As mercapto groups form even more stable conjugates with certain types of nanoparticle than most other chemical groups (amino $-NH_2$ groups, for example), it is another aspect of the invention to provide a method for preparing stable nanoparticle conjugates by multivalent conjugation of mercapto-substituted flexible hydrophilic polymers, mercaptodextrans for example, to nanoparticles, preferably in a manner which controls the number of molecules conjugated to each nanoparticle.

Accordingly, the invention provides a nanoparticle conjugate comprising a nanoparticle conjugated to a functionalised flexible hydrophilic polymer via a plurality of mercapto groups. The invention also provides methods for producing such nanoparticles, as hereinbefore described.

In effect the method of the invention enables the synthesis of the entire surface of the nanoparticle conjugate before its formation by conjugation. This has the significant advantage that if the surface is constructed at high concentration, the number of functional molecules substituted into the surface (before conjugation to the surface of the nanoparticle) can be determined directly, and any purification steps can be carried out using conventional techniques that give a high yield.

The flexible hydrophilic polymer is preferably selected from polysaccharides, polyethylene glycols, polyvinyl alcohols, polyacrylic acids, polyacrylamides, polyamides (including polyaminoacids), polycarboxylated polymers and copolymers thereof. Examples of suitable polysaccharides include dextran, agarose, carrageenan, alginic acid, starch and cellulose. Dextran and derivatives thereof, such as aminodextran, carboxydextran and carboxymethyldextran are particularly preferred.

The plurality of substituents capable, optionally after deprotection, of binding to nanoparticles may effect such binding chemically, electrostatically, hydrophobically or by a combination thereof. The flexible hydrophilic polymer is preferably provided with pendant substituents with such capability. Such substituents preferably comprise a conjugation group (for conjugation to the nanoparticle) selected from sulphides (-S-), asymmetrical or symmetrical

disulphides (–S-S-), selenides (-Se-), diselenides (-Se-Se-), mercapto (thiol, sulphydryl, -SH), nitrile (-CN), isonitrile, nitro ($-NO_2$), amino (NH_2), selenol (-SeH), trivalent phosphorous compounds, isothiocyanate, xanthate, thiocarbamate, phosphine, thioacid (-COSH) or dithioacid (-CSSH) and thioester (-COS-). Particularly preferred conjugation groups in this respect include mercapto (thiol, sulphydryl, -SH) and disulphide (-S-S-).

The number, per molecule of flexible hydrophilic polymer, of substituents capable, optionally after deprotection, of binding to the nanoparticles is greater than one, preferably greater than two, more preferably greater than about three, still more preferably greater than about five, most preferably greater than about ten.

The functional molecules may, for example, find applications in biomolecular assays, as ligands for targeting biochemical receptors, or as therapeutic or pharmacological agents. Examples of suitable functional molecules include chelating agents, haptens, biotin, avidin, streptavidin, antibodies including monoclonal antibodies, Fab' fragments, Fab fragments, enzymes, enzyme cofactors, hormones, specific carbohydrates, polynucleotides, lectins, or chimaeric or fusion molecules derived from two or more of these molecules.

The nanoparticle may comprise a metal, for example Au, Ag or a bimetallic composite thereof. Other example of suitable nanoparticle materials include semiconductors such as the sulphides and selenides of Zn, Cd, Pb, Sn, Hg, Al, Ga, In, Ti, Si, Ag, Fe, Fe, Ni and Ca. Preferred semiconductor nanoparticles include CdSe, ZnSe, CdTe, InP, InAs, PbSe, PbS and CdS. Metal oxide nanoparticles such as iron oxide may also be used. The nanoparticle may have a

core-shell structure, in which case the shell may be a metal, semiconductor or metal oxide, and the core may be a metal, semiconductor, metal oxide or metalloid oxide. In some cases the core material may be chosen to render the nanoparticle conjugates responsive to a magnetic field.

The present invention will now be described in more detail, by way of example only, with reference to the following Figures, in which:

Figure 1 illustrates a schematic representation of a monovalent conjugation method in which oligonucleotides with a terminal mercapto group are conjugated to gold nanoparticles;

Figure 2 illustrates a comparison between the dissociation of monovalent and multivalent conjugates;

Figure 3 illustrates a schematic representation of a simple multivalent (divalent) conjugation method based on dihydrolipoic *acid (J. Am. Chem. Soc., 122 (2000) 12142-12150*). Following conjugation the COOH groups of the conjugate are covalently linked to a protein molecule (avidin);

Figure 4 illustrates the structural formula of aminotrithiolate that has been used for the multivalent (trivalent) conjugation of antibodies to metallic nanoparticles. This multivalent molecule binds so tightly to silver nanoparticles that aminodextrans, multivalently conjugated to the nanoparticles by a plurality of primary amine groups, are displaced by it (U.S. Pat No. 5,945,293 (1999));

Figure 5 illustrates the schematic representation of an entrapment method of conjugation based on mercaptopropyltrimethoxy silane. The mercapto groups bind to the nanoparticle and the alkoxy groups point outwards where they are available for cross-linking to each other and to other silanes. In this representation

the other silanes have primary amine groups that can be covalently linked to binding molecules such as antibodies;

Figure 6 illustrates a comparison between the equilibria that obtain in reagent-limited immunoassays when nanoparticles with high and low functional affinity are used. The high functional affinity nanoparticles are based on those used in an actual immunoassay for trinitrotoluene (TNT) (*Anal. Chem. 74 (2002) 841-847*);

Figure 7 illustrates a comparison of the results of when nanoparticle conjugates with the same size but different numbers of binding molecules are used to enhance the signal in SPR assays. Particles with large numbers of binding molecules provide less signal enhancement than particles with a low number of binding molecules;

Figure 8 illustrates a schematic representation of expression *analysis (BioEssays, 18 (1996) 427-431 and BioTechniques, 33 (2002) 620-630)* emphasizing that nanoparticle conjugates with only one oligonucleotide per particle are essential for comparison on a single microarray;

Figure 9 illustrates a summary of the method of the present invention for conjugating a controlled number of molecules to a nanoparticle;

Figure 10 illustrates a schematic representation showing how particles with a large number of binding molecules reduce the dynamic range of an assay by excluding other nanoparticle conjugates from the separation phase;

Figure 11 illustrates a schematic representation showing that large particles with a low number of binding molecules have slow kinetics;

Figure 12 illustrates a schematic representation showing how large particles lead to ambiguity and hence to insensitivity;

Figure 13 is a graphical representation of the UV/vis Spectrum of protected mercaptodextran substituted with DNP haptens. The number of DNP haptens can be determined from the absorbance at 360 nm and the number of mercapto groups from the increase in absorbance at 343 nm when DTT is added;

Figure 14 illustrates the step of deprotection of haptenylated mercaptodextran molecule with DTT (before conjugation to nanoparticles the mercaptodextran is purified by gel-exclusion chromatography);

Figure 15 illustrates a schematic representation of the self-assembly of haptenylated mercaptodextrans to gold nanoparticles;

Figure 16 is a graphical representation of the UV/vis spectra showing the effect of conjugating different numbers of haptenylated mercaptodextrans to gold nanoparticles. The numbers on the spectra correspond to the number of dextran molecules per particle. When there are insufficient dextrans, the particles flocculate on addition of PBS as indicated by the decrease in absorbance at 520 nm;

Figure 17 illustrates a schematic summary of a paramagnetic microbead immunoassay for DNP-gold nanoparticle conjugates;

Figure 18 is a graphical representation of the UV/vis spectra showing the absorbance changes that occur when different amounts of antibodies bound to paramagnetic microbeads are rotated with a nanoparticle conjugate solution; and

Figure 19 is a graphical representation of the absorbance at 520 nm for a gold nanoparticle conjugate solution against the amount of antibodies bound to paramagnetic microbeads added and removed by magnetic precipitation.

Figures 1 to 8 have already been described and discussed with reference to the prior art.

In the following discussion, reference is made, for convenience, to nanoparticle conjugates wherein the flexible hydrophilic polymer is a dextran or a dextran derivative and wherein the plurality of conjugation substituents capable of binding to nanoparticles are mercapto substituents, or protected substituents which provide, on deprotection, the same type of conjugation as would be provided by mercapto substituents. However, it should be recognized that other types of flexible hydrophilic polymer and other types of conjugation substituent are contemplated herein and are within the scope of the invention and the ensuing discussion.

Referring to Figure 9A, one method of the invention, involving the synthesis of mercaptodextrans substituted with a controlled number of one or more functional molecules, is schematically illustrated. This step is carried out under conditions that allow the number of functional molecules substituted into the dextran to be accurately determined. In Figure 9B the substituted mercaptodextrans are multivalently conjugated to the nanoparticles, by a process of self-assembly, in which the number of dextran molecules per particle is determined by the relationship between dextran size and particle size.

The invention provides a method (summarized in Figure 9) for synthesizing a surface polymer with a known and controlled number of functional

molecules at high concentration prior to conjugation. This surface polymer is conjugated to the nanoparticles by a process of self-assembly in which the sulphur atoms in a plurality of mercapto groups become chemically bound to the nanoparticles. The number of polymer molecules conjugated to each nanoparticle is determined by the size of the polymer and the size of the nanoparticle. This is an example of a method preparing nanoparticle conjugates, in which a polymer with a known number of functional molecules is conjugated to particles by a plurality of electrostatic, hydrophobic or chemical interactions, such that the number of polymer molecules per particle is determined by the size of the polymer and size of the particle. One of the main advantages of nanoparticles is that they have dimensions that are similar to the molecules that are used in biomolecular assays as shown in Table I:

TABLE I

Entity	Approximate Dimensions (nm)
Glucose	0.5
Biotin	0.7
DNA Helix (25 bases)	8.5 x 2
Antibody (Immunoglobulin G)	4
Glucose Oxidase	8
Semiconductor nanoparticles	2-10
Metallic nanoparticles	< 100
Animal Viruses	22 - 120
Bacteria	500 – 1200
Mitochondrion	600
Chloroplasts	5000
Human Cells	10,000

As the particle size increases the number that can be accommodated by the separation phase in a biomolecular assay decreases as shown in Figure 10; this reduces the dynamic range and sensitivity of an assay. The increase in surface area of large particles promotes non-specific adsorption even when they are coated with a resistant surface. The rate of binding reactions decreases due to slower diffusion and because the particles act as barrier between biomolecular interactions. The latter effect is particularly pronounced when a limited number of binding molecules are conjugated to large particles as shown in Figure 11. The superior optical properties of large particles must be weighed against these

drawbacks. High functional affinity and large particle size are not always a problem, however, and in two-step reagent excess assays they may even confer an advantage (*Anal. Chem., 73 (2001) 2254-2260*). Large particles with a large number of binding sites can enhance both kinetics and sensitivity provided the separation phase has a low density of binding sites, but when the binding site density is high large particles lead to ambiguous results as shown in Figure 12.

The invention provides for a method of conjugating a known number of molecules that can participate in biomolecular and other applications to nanoparticles. In one such method the molecules are covalently attached to a polysaccharide that is also substituted with a plurality of pendant mercapto groups. The polysaccharide is conjugated to the nanoparticles by a multivalent method in which the sulphur atoms of the mercapto groups are chemically bound to the nanoparticles.

The invention provides in one of its aspects a two-step method for preparing nanoparticle conjugates. In the first step of the method a polysaccharide is substituted with a predetermined number of two or more molecules, one of which has a mercapto group, or a chemical group that can be converted to a mercapto group. The first step is carried out under conditions that allow the number and type of molecules substituted into the polysaccharide to be accurately determined. In the second step of the method the substituted polysaccharide is conjugated to the nanoparticles by a process of self-assembly in which the sulphur atoms of the mercapto groups become chemically bound to the particles. The second step may be carried out in such a way that the number of substituted

polysaccharides conjugated to each nanoparticle is controlled by the relationship between the size of the polysaccharide and the size of the particle.

Described herein is a method for conjugating a known number and type of one or more functional molecules to a nanoparticle (Figure 9). For the purpose of the invention the term nanoparticle (nanoparticle) refers to particles with diameters of preferably less than about 100 nm. In the first part of the method the said functional molecules are covalently attached to a flexible hydrophilic dextran polymer that is also substituted with mercapto groups or protected mercapto groups. This part of the method is preferably carried out under conditions that allow the number and type of molecules substituted into the polymer to be accurately determined. In the second part of the method the substituted polymer is conjugated to the nanoparticles by a process of self-assembly, in which the sulphur atoms of the mercapto groups are chemically bound to the particles. The second part of the method is carried out in such a way that the number of polymer molecules conjugated to each nanoparticle is controlled by the relationship between dextran size and particle size.

The flexible hydrophilic polymer into which molecules are to be substituted is preferably derivatized with primary amines that can be covalently attached to substituent molecules. The polymer is preferably an aminopolysaccharide and preferably an aminodextran, where the word dextran refers to any branched polysaccharide of D-glucose, regardless of the branch point of the repeating unit; i.e., 1→2, 1→3, 1→4, etc. Aminodextrans are dextrans that have been derivatized with primary amine (NH_2) groups. Methods for preparing aminodextrans include reductive amination of periodate oxidized dextran

(Biosens. Bioelectron., 11 (1996) 579-590), reaction of activated dextrans with mono-protected diamines followed by removal of the protecting group (Makromol. Chem., 192 (1991) 673-677), and reaction of excess diaminoalkanes with periodate oxidized dextran (Bioconjugate Chem., 10 (1999) 1090-1106).

In order to produce nanoparticle conjugates that can be used in biomolecular assays and other applications aminodextrans are substituted either directly with functional molecules that can participate in the said applications, or indirectly with molecules possessing chemical groups that can be linked to functional molecules that can participate in the said applications. The direct substitution of molecules into aminodextrans is preferably accomplished by activating the molecules that are to be substituted, or derivatives thereof, with a reactive group. Examples of reactive groups are succinimidyl or sulfosuccinimidyl esters, isothiocyanates and sulfonyl chlorides. Examples of activated molecules are succinimidyl 6-(biotinamido)hexanoate, fluorescein-5(6)-carboxamido-caproic acid NHS and atrazine NHS ester (Biosens. Bioelectron, 12 (1997) 277-286). The indirect substitution of molecules into the aminodextrans is accomplished by activating the primary amines with a bifunctional reagent. Examples of bifunctional reagents are suberic acid bis (NHS ester), 6-(iodoacetamide)caproic acid NHS ester, succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), 3-maleimidobenzoic acid NHS (MBS), γ-maleimidobutyric acid NHS (GMBS), ε-maleimidocaproic acid NHS (EMCS), β-maleimidopropionic acid NHS (BMPS), 6-(4-azido-2-nitrophenylyamino)hexanoic acid NHS ester, 4-

azidobenzoic acid NHS ester and 4-azido-2,3,5,6-tetrafluorobenzoic acid NHS ester.

For the purpose of conjugating the substituted dextran molecules to nanoparticles in the second part of the method, the aminodextrans are also substituted with mercapto (thiol, sulphydryl, -SH) groups, or chemical groups that can be converted to mercapto groups by removal of a protecting group. Mercaptodextrans have been prepared previously for use as chelating agents (Acta Pharmacologica. Sinica, 11 (1990) 363-367) and for coating macroscopic gold surfaces with dextrans (Biomaterials, 21 (2000) 957-966). Mercapto groups can be substituted into aminodextrans by reaction with reagents such as 2-iminothiolane. Chemical groups that can be converted to a mercapto groups by removal of a protecting group can be substituted into aminodextrans with reagents such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), N-hydroxysuccinimide s-acetylthioacetic acid (SATA) and S-acetylmercaptosuccinic anhydride (SAMSA). SPDP can be deprotected with dithiothreitol (DTT) or tris-(2-carboxyethyl)phosphine (TCEP), and SATA and SAMSA can be deprotected with 50 mM hydroxylamine. In some manifestations of the invention it is important that SATA and SAMSA (unlike SPDP) do not react with mercapto groups prior to deprotection. Further examples of bifunctional reagents are described in texts such as The Pierce Handbook and Chemistry Of Protein Conjugation And Cross-Linking [Wong, S.S.] CRC Press, Inc., Boca Raton, Fl, (1991).

A particular advantage of the invention is that the number and type of molecules substituted into the aminodextran polymer may be finely controlled.

Aminodextrans can be substituted with a broad numerical distribution of molecules by reacting a fixed amount of the reactive molecule with a fixed amount of aminodextran, such that the molarity of primary amines in the aminodextran exceeds the molarity of reactive molecules. Aminodextrans with a narrow numerical distribution of molecules are prepared by reacting a fixed ratio of two or more reactive molecules simultaneously with a fixed amount of aminodextran, such that the molarity of the reactive molecules exceeds the molarity of primary amines in the aminodextran.

The substitution of molecules into aminodextrans is preferably carried out under conditions that allow the number and type of the said molecules to be determined accurately. For this purpose it is advantageous if the molecules are substituted into the aminodextrans at relatively high concentrations such that the degree of substitution can be accurately determined by simple methods such as UV/vis spectroscopy. In the context of the invention relatively high concentrations of aminodextrans preferably refer to concentrations equal to or exceeding 5μM, preferably 8μM, more preferably 10μM, for example 12μM or 15μM. For the purpose of accurate determination it is also advantageous if the polymer is prepared in the absence of interference from the nanoparticles. Preparation at relatively high concentration also facilitates purification and increases the yield of the substituted dextran product.

In the second part of the method the substituted mercaptodextrans prepared in the first part of the method are conjugated to the nanoparticles. In the context of the invention nanoparticle may refer to a metal, metal oxide or semiconductor particle that is preferably less than about 100 nm in diameter.

Conjugation is carried out by a process of self-assembly in which the sulphur atoms of mercapto groups or protected disulphide bonds in the dextran become chemically bound to the nanoparticle. When the dextran is substituted with a protected disulphide bond mercapto groups may be generated by spontaneous fission of the said bond on contact with the nanoparticle (J. Am. Chem. Soc., 124 (2002) 5811-5821) or by chemical fission of the disulphide with a reducing agent such as DTT or TCEP or a nucleophile such as hydroxylamine (Biochem. J., 173 (1978) 723-737; Anal. Biochem., 132 (1983) 68-73). When the mercapto groups are generated by chemical fission of a disulphide bonds the substituted dextran may be purified by gel-exclusion chromatography prior to conjugation in order to remove small molecules that are capable of binding to the nanoparticles.

Another advantage of the invention is that the number and type of molecules conjugated to each nanoparticle may be finely controlled. When a fixed number of nanoparticles are titrated with different amounts of a given mercaptodextran, the minimum number of mercaptodextran molecules required to stabilize the nanoparticles depends on the size of the mercaptodextran molecules and the size of nanoparticles. The size of a mercaptodextran molecule determines the extent to which it can cover the surface area of a nanoparticle; size is related to the molecular weight (MW) of the dextran, but other factors such as the amount of polymer branching, the number and type of the functional molecules and the number of mercapto groups are also involved. If the size of the nanoparticle is appropriately matched to the size of the mercaptodextran the number of mercaptodextran molecules that can be accommodated by each nanoparticle is limited to one. When this happens the number of functional molecules conjugated

to each nanoparticle is equal to the number of functional molecules in one molecule of the mercaptodextran. When the nanoparticle can accommodate more than one mercaptodextran molecule the number of molecules conjugated to each particle will be a multiple of the number of functional molecules in one molecule of the mercaptodextran.

The following Examples further illustrate the invention:

Example 1

This Example describes the production of protected mercaptodextrans with a known number of haptens. It is possible to produce dextrans substituted with a mean value of one hapten per molecule of dextran by adding a fixed amount of reactive hapten to a fixed amount of aminodextran as determined by titration. The actual number of haptens per molecule of dextran, however, is a Poisson distribution about this mean value because of concentration differences that occur during the reaction. A more efficient way to synthesize dextrans with a well-defined number of haptens is by ratiometric reaction of a fixed amount of aminodextran with a fixed ratio of reactive hapten to reactive mercapto compound. To prepare protected mercaptodextrans with one hapten per molecule 6(2,4-dinitrophenylamino)-1-aminohexanoic acid [N-hydroxysuccinimide ester] (DNPAH-NHS) and SPDP were dissolved in dry DMSO to final concentrations of 7.2 mM and 0.116 M respectively. This solution (0.2 ml) was added, dropwise with stirring to 10 mg of aminodextran (MW 70,000; 16.2 primary amines per molecule as determined by the phthalaldehyde method described in: Makromol. Chem., 192 (1991) 673-677) dissolved in 5 ml of 50 mM (pH unadjusted) bicarbonate solution. After standing overnight at 4°C in darkness, the solution was

loaded onto a PD-10 column (Sephadex G-25, from Amersham Pharmacia Biotech) and eluted with tris buffered saline (TBS). A UV/Vis spectrum of the haptenylated dextran eluted form the PD-10 column is shown in Figure 13. The spectrum corresponds closely to the spectrum of dinitrophenol and 2-pyridyldisulphide, with a small additional amount of absorbance at short wavelengths due to scattering by the dextran. From the absorbance at 360 nm (1.74×10^4 M^{-1} cm^{-1}; Anal. Biochem. 177 (1989) 392-395) and the increase in absorbance at 343 nm (8.08×10^3 M^{-1} cm^{-1}; Biochem. J., 173 (1978) 723-737) on adding dithiothreitol (DTT) the ratio of dinitrophenyl (DNP) hapten to protected mercapto groups was determined to be 16:1; this corresponds closely to one hapten per molecule of dextran. The proposed structure of the haptenylated dextran is shown in Figure 14. This method produces dextrans with a very narrow distribution of haptens because degree of substitution depends on the ratio of the reactive hapten to the reactive mercapto compound, and not on their absolute concentrations, which may vary during the reaction. Protected mercaptodextrans with more than one hapten per molecule can be synthesized by altering the ratio of reactive hapten to reactive mercapto compound. Protected mercaptodextrans substituted with reactive derivatives of molecules other than DNP can be synthesized by similar methods, and by extension known amounts of more than two molecules (e.g. DNP, biotin and pyridyldithiopropionate) can also be substituted into aminodextrans.

Example 2

This Example describes the production of protected mercaptodextrans with a known number of proteins. Where the protein that is to be conjugated does not

contain a suitable mercapto group it is thiolated with 2-imminothiolane, or with SPDP followed by reductive deprotection of the disulphide and gel-exclusion chromatography. The latter is preferred because it minimizes the possibility of disulphide crosslinking and allows the average number of mercapto groups substituted into each protein molecule to be determined from the amount of pyridinedithione chromophore released on reduction with DTT. Introduction of a mercapto groups into antibodies is carried out by adding 75 µl of 13.5 mM of 2-imminothiolane [HCl salt] in PBS to a stirred solution of 10 mg of antibody (IgG) in 2 ml of PBS. After slant rotating for 1 hour at room temperature the thiolated antibody is purified by dialysis against PBS, or by gel-exclusion chromatography on Sephadex G-25. Fab' fragments which have a single antigenic binding site are particularly useful in the context of the invention because they allow the number of binding sites per nanoparticle conjugate to be limited to one. Fab' fragments are prepared by proteolytic cleavage of antibodies with pepsin followed by reduction with 2-mercaptoethylamine (J. Biochem. 92(1982)1413-1424). They contain a single mercapto group, which is remote from the single antigen binding site, and therefore it is unnecessary to introduce a mercapto group chemically. Protected mercaptodextrans with one maleimide group per molecule of dextran are prepared by a method similar to Method 1, except that SMCC is used in place of DNPAH-NHS, and SATA is used in place of SPDP. The ratio of mercapto groups to maleimide groups in the dextran is determined by reacting the protected mercaptodextran with deprotected fluorescein SAMSA (Molecular Probes, Eugene, Oregon, USA) and reacting the deprotected mercaptodextran with 5,5'-dithiobis-(2-nitrobenzoic acid) (DNTB, Ellman's reagent; Anal. Biochem.; 101

(1980) 442-448). The number of maleimide groups is determined from the fluorescence at 520 nm and the number of mercapto groups is determined from the absorbance of the 410 nm (1.36×10^4 M^{-1} cm^{-1}). In order to ensure that there is only one protein molecule per molecule of dextran the latter is added dropwise to an excess of thiolated protein (5 x the molarity of maleimides in the dextran) in pH 6.0, 0.1 M phosphate buffer and slant rotated for 30 minutes. At the end of this time the dextran protein conjugates are purified on Sepharose 4B. Protected mercaptodextrans with a known number of oligonucleotides are prepared by similar methods except that oligonucleotides with a terminal mercapto group are used instead of thiolated proteins

Example 3

This Example describes the production of gold nanoparticle conjugates with a known number of haptens. The process of self-assembly used to conjugate substituted mercaptodextrans to nanoparticles is shown in Figure 15. A fixed amount of nanoparticles is mixed with different amounts of deprotected mercaptodextran dissolved in water and allowed to stand for 1 hour. Phosphate buffered sodium chloride is added to give a final concentration equivalent to PBS (15 mM Na Phosphate, 0.15 M NaCl). When the amount of dextran was too low to stabilize the particles they flocculated on addition of PBS as shown in Figure 16. The minimum number of haptenylated mercaptodextrans required to stabilize the particles depends on the diameter of the particles. When the particles have a diameter of 12 nm a minimum of four haptenylated mercaptodextrans (as prepared in Method 1) are required. Because the number of haptens per molecule of mercaptodextran molecule and number of mercaptodextrans per particle are

known the number of haptens per nanoparticle conjugate can be calculated. In the example given here there is one hapten per mercaptodextran molecule and four mercaptodextran molecules per nanoparticle. Therefore the number of haptens per nanoparticle conjugate is four. By altering the size of the particles the number of mercaptodextrans per nanoparticle, and hence the number of haptens per nanoparticle conjugate, can be varied in such a way that a known number of molecules can be conjugated to each nanoparticle.

Example 4

This Example describes the reactivity of mercaptodextran nanoparticle conjugates in biomolecular assays. The biomolecular reaction of haptenylated nanoparticle conjugates to antibody-coated paramagnetic microbeads is summarised in Figure 17. DNP-gold-nanoparticle conjugates were slow tilt rotated with paramagnetic beads coated with the corresponding antibody (anti-DNP) in PBS that contained 1 mg ml^{-1} BSA and 0.05% Tween-20. After rotating for ten minutes the beads were magnetically precipitated and the UV/vis spectrum of the supernatant was recorded. Figure 18 shows how the absorbance spectrum changed as the amount of beads increased; no change was observed in control experiments when rotation was carried out in the presence of 10 μM DNP, or when beads coated with non-specific (anti-mouse) antibodies were used. When magnetically precipitated antibody coated beads were resuspended and rotated with 10 μM DNP, bound nanoparticles were released into solution. These results show that gold nanoparticles coated with haptenylated mercaptodextran bind specifically to the corresponding antibody. Results were unaffected by BSA and Tween-20 showing that the haptenylated mercaptodextrans were not displaced.

This method of conjugation is suitable for most haptens, and other molecules, including oligonucleotides and antibodies. Picomolar amounts of antibody were detected, as shown in Figure 19.

CLAIMS

1. A method for the preparation of nanoparticle conjugates comprising:

a) providing a first reagent comprising a flexible hydrophilic polymer;

b) providing a second reagent comprising at least one functional molecule capable of being substituted into the flexible hydrophilic polymer;

c) providing a third reagent comprising nanoparticles;

d) contacting the first reagent with the second reagent for a period of time and under conditions effective to allow substitution of the at least one functional molecule into the flexible hydrophilic polymer;

e) before, during and/or after step d) providing the flexible hydrophilic polymer with a plurality of substituents capable, optionally after deprotection, of binding to the nanoparticles to provide an intermediate product comprising the flexible hydrophilic polymer substituted with the at least one functional molecule and a plurality of substituents capable, optionally after deprotection, of binding to the nanoparticles;

f) if necessary, deprotecting the plurality of substituents capable of binding to the nanoparticles; and

g) contacting the, if necessary deprotected, intermediate product of step e) with the third reagent for a period of time and under conditions effective to allow binding of the, if necessary deprotected, intermediate product with the nanoparticles to provide the nanoparticle conjugates

wherein the number of functional molecules conjugated per nanoparticle in the final step is controlled by at least one of:

* controlling, by means of suitable selection of reagents and reaction conditions, the number of functional molecules substituted into the flexible hydrophilic polymer in step d);

* controlling, by means of suitable selection of reagents and reaction conditions, the number of optionally protected substituents capable of binding to the nanoparticles substituted into the flexible hydrophilic polymer in step e); and

* controlling, by means of suitable selection of reagents and reaction conditions, the number of intermediate product molecules binding to the nanoparticles in step g).

2. A method according to claim 1 wherein control of the number of functional molecules per nanoparticle is achieved by at least one of:

* selecting the relative sizes of the flexible hydrophilic polymer and the nanoparticle to control the number of molecules of flexible hydrophilic polymer, and therefore of optionally deprotected intermediate products, which can be accommodated on the surface of the nanoparticle;

* selecting the relative concentrations of the first and second reagents in step d) to control the number of functional molecule(s) substituted into each molecule of flexible hydrophilic polymer; and

* selecting the number per molecule of flexible hydrophilic polymer of substituent molecules capable, optionally after deprotection, of binding to the nanoparticles.

3. A method according to claim 1 or claim 2 comprising determining at least approximately the desired number of, if necessary deprotected, intermediate product molecules to be bound to each nanoparticle in step g) and selecting the relative size of the flexible hydrophilic polymer and the nanoparticle such that the number of, if necessary deprotected, intermediate product molecules which can be accommodated on the surface of each nanoparticle at least approximately matches the desired number.

4. A method according to any one of claims 1 to 3 comprising determining at least approximately the desired number of functional molecule(s) to be substituted into each molecule of flexible hydrophilic polymer in step d) and selecting accordingly the reagent concentrations and reaction conditions in step d).

5. A method according to any one of claims 1 to 4 comprising determining at least approximately the desired number of substituent molecules capable, optionally after deprotection, of binding to the nanoparticles to be substituted into each molecule of flexible hydrophilic polymer in step e) and selecting accordingly the reagent concentrations and reaction conditions in step e).

6. A method according to any one of claims 1 to 5 wherein the relative size of the flexible hydrophilic polymer and the nanoparticle are selected to be effective to allow binding in step g) of a controlled number of the, if necessary deprotected, intermediate product molecules with the nanoparticles.

7. A method for the preparation of a nanoparticle conjugates comprising:

i) providing a first reagent comprising a flexible hydrophilic polymer having a plurality of substituents capable, optionally after deprotection, of binding to a nanoparticle;

ii) providing a second reagent comprising one or more functional molecules suitable for binding to target molecules, optionally in a biomolecular assay, and capable of being substituted into the flexible hydrophilic polymer;

iii) providing a third reagent comprising nanoparticles capable of binding to the plurality of substituents of the flexible hydrophilic polymer;

iv) contacting the first reagent with the second reagent for a period of time and under conditions effective to allow the substitutionof the at least one functional molecule into the flexible hydrophilic polymer and provide an intermediate product comprising the flexible hydrophilic polymer substituted with the at least one functional molecule; and

v) contacting the intermediate product of step iv) with the third reagent for a period of time and under conditions effective to allow binding of the intermediate product with the nanoparticles to provide the nanoparticle conjugate.

8. A method according to any one of claims 1 to 7 wherein the number of functional molecules substituted into the flexible hydrophilic polymer in the intermediate product is determined before the step of contacting the intermediate product with the third reagent.

9. A method according to any one of claims 1 to 8 wherein the number of functional molecules per molecule of the flexible hydrophilic polymer in the intermediate product is determined before the step of contacting the intermediate product with the third reagent.

10. A method according to any one of claims 1 to 9 including the step of determining the number of intermediate product molecules bound to the nanoparticles in the nanoparticle conjugates.

11. A method according to any one of claims 1 to 10 including the step of determining the number of intermediate product molecules bound per nanoparticle in the nanoparticle conjugates.

12. A method according to any one of claims 1 to 11 including the step of determining at least approximately the average size of the nanoparticles and selecting the hydrophilic flexible polymer to be of an overall size (considering at least one or more of molecular weight, chain length and degree of chain branching) such that the nanoparticle conjugate is able to accommodate a number z of flexible hydrophilic polymer molecules around its surface.

13. A method according to claim 12 wherein z is less than about 4.

14. A method according to claim 13 wherein z is 1.

15. A method according to claim 12 wherein z is greater than about 20 and/or greater than about 15 and/or greater than about 10.

16. A method for producing a nanoparticle conjugate comprising:

I) providing a nanoparticle having a surface area x;

II) providing a flexible hydrophilic polymer having a chain length and degree of branching such that a molecule of the polymer has the capacity, when suitably conformed, to envelop a surface area x/y;

III) substituting the polymer with a plurality of conjugation substituents capable of binding to the nanoparticle and with at least one functional molecule capable of imparting a function in a biomolecular assay or other application to the nanoparticle conjugate; and

IV) conjugating approximately y molecules of the polymer to the nanoparticle via the said plurality of conjugation substituents;

wherein the flexible hydrophilic polymer is selected with regard to the variable y to provide a nanoparticle conjugate having an at least approximately predetermined number of flexible hydrophilic polymer molecules per nanoparticle.

17. A nanoparticle conjugate derivable from a method according to any one of claims 1 to 16 which comprises a single molecule of intermediate product conjugated to each nanoparticle.

18. A nanoparticle conjugate derivable from a method according to any one of claims 1 to 16 which comprises two molecules of intermediate product conjugated to each nanoparticle.

19. A nanoparticle conjugate derivable from a method according to any one of claims 1 to 16 which comprises from about 3 to about 5 molecules of intermediate product conjugated to each nanoparticle.

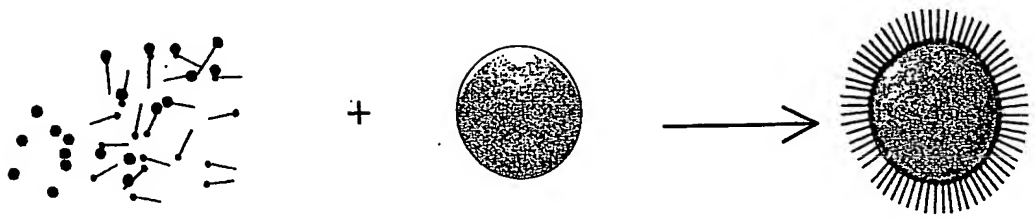
20. A nanoparticle conjugate derivable from a method according to any one of claims 1 to 16 which comprises a nanoparticle conjugated to a flexible hydrophilic polymer having at least one functional molecule thereon, the conjugation being provided by multiple mercapto groups.

21. A nanoparticle conjugate comprising a nanoparticle conjugated to a functionalised flexible hydrophilic polymer via a plurality of mercapto groups.

ABSTRACT

The present invention provides a method for the preparation of nanoparticle conjugates comprising: protocols for synthesizing intermediate product molecules by introducing known numbers of two or more substituents into a flexible hydrophilic polymer, where one substituent is capable, optionally after deprotection, of binding to a nanoparticle, and where the other substituents are capable of participating in analytical or other applications; protocols for contacting the intermediate product molecules with nanoparticles for a period of time and under conditions effective to allow the binding of a known number of the intermediate product molecules to each nanoparticle to provide the nanoparticle conjugate.

Figure 1



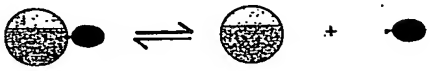
Key:

Alkythiol Oligonucletide

Gold Nanoparticle

Figure 2

A) Dissociation of a monovalent NPC



$$K_d = \frac{[\quad][\quad]}{[\quad]}$$

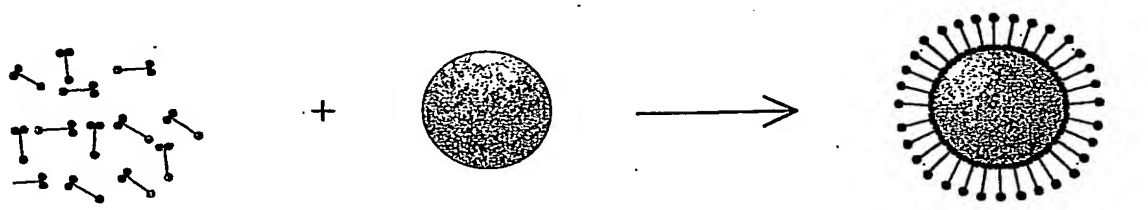
B) Dissociation of a multivalent NPC

$$K_t = \frac{[\quad][\quad]}{[\quad][\quad][\quad][\quad]}$$

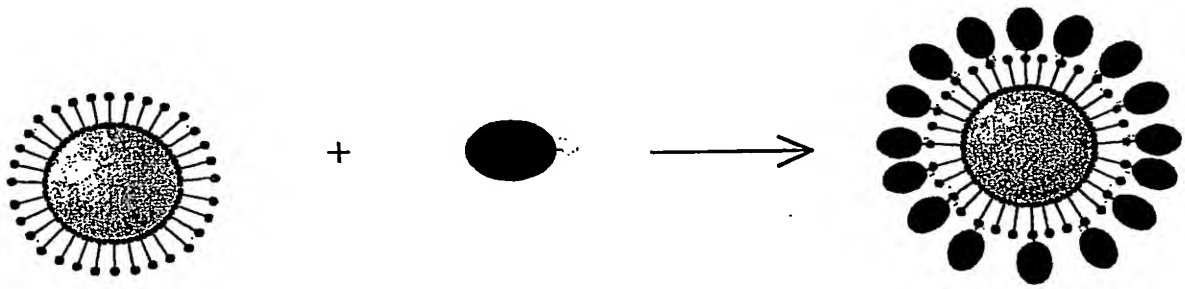
Where $K_t << K_d$

Figure 3

A) Divalent conjugation



B) Covalent attachment of binding molecules



Key:

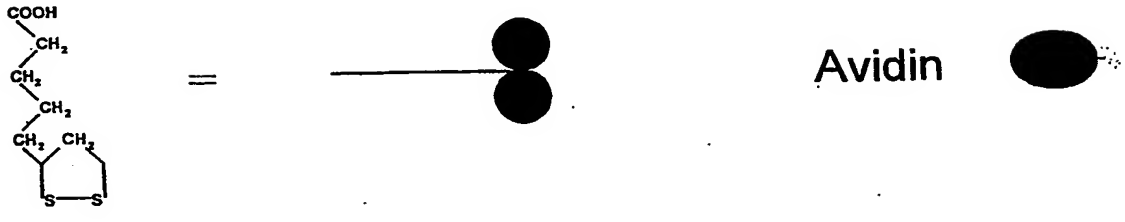


Figure 4

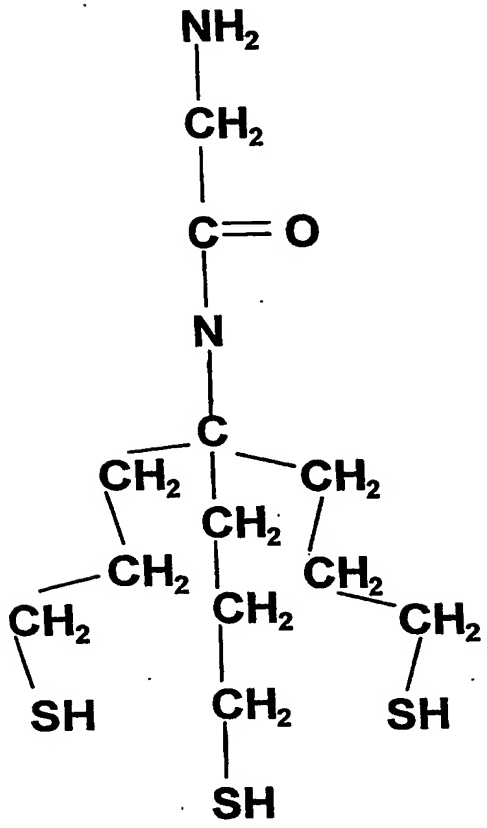
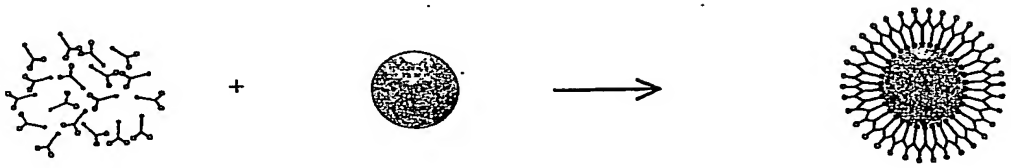
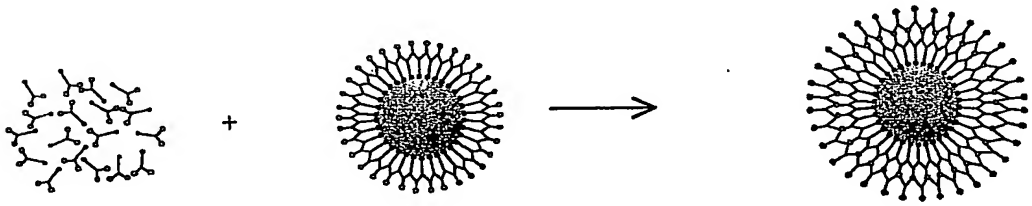


Figure 5

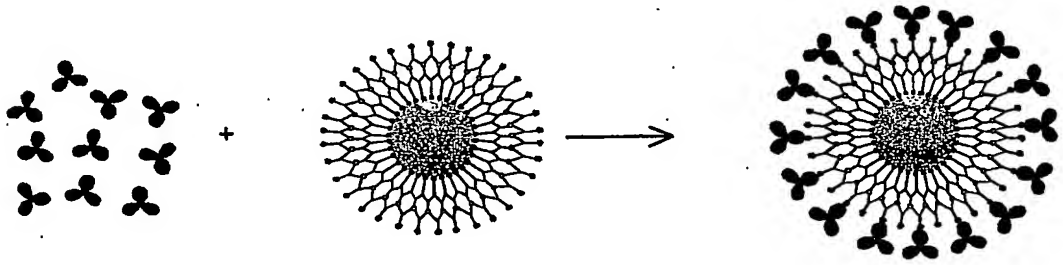
A) Monovalent conjugation



B) Polymerization (entrapment) and functionalization



C) Covalent attachment of binding molecules



Key:

Mercaptopropyltrimethoxy Silane

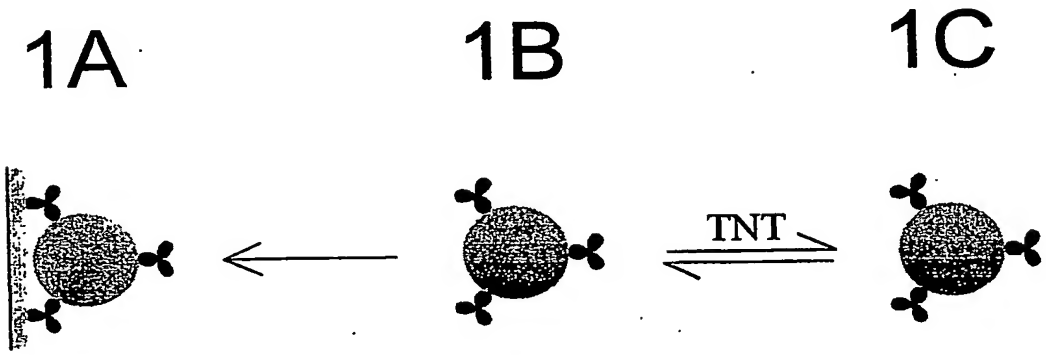
Gold Nanoparticle

Aminopropyltrimethoxy Silane

Antibody

Figure 6

A) High Functional Affinity - Low Sensitivity



B) Low Functional Affinity - High Sensitivity

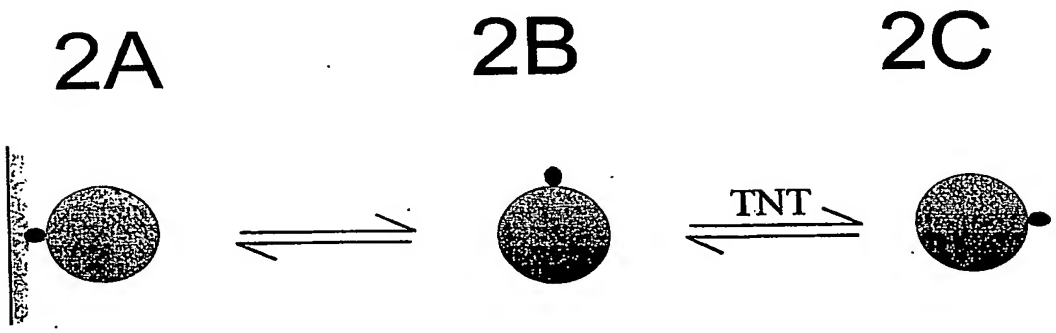
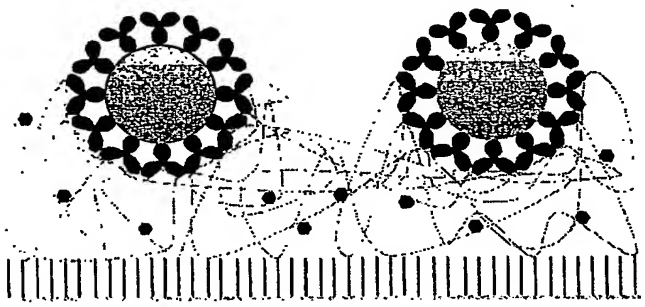


Figure 7

A) High Binding Molecules - Low Signal Strength



B) Low Binding Molecules - High Signal Strength

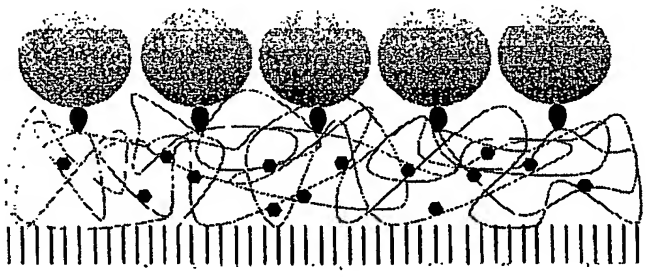


Figure 8

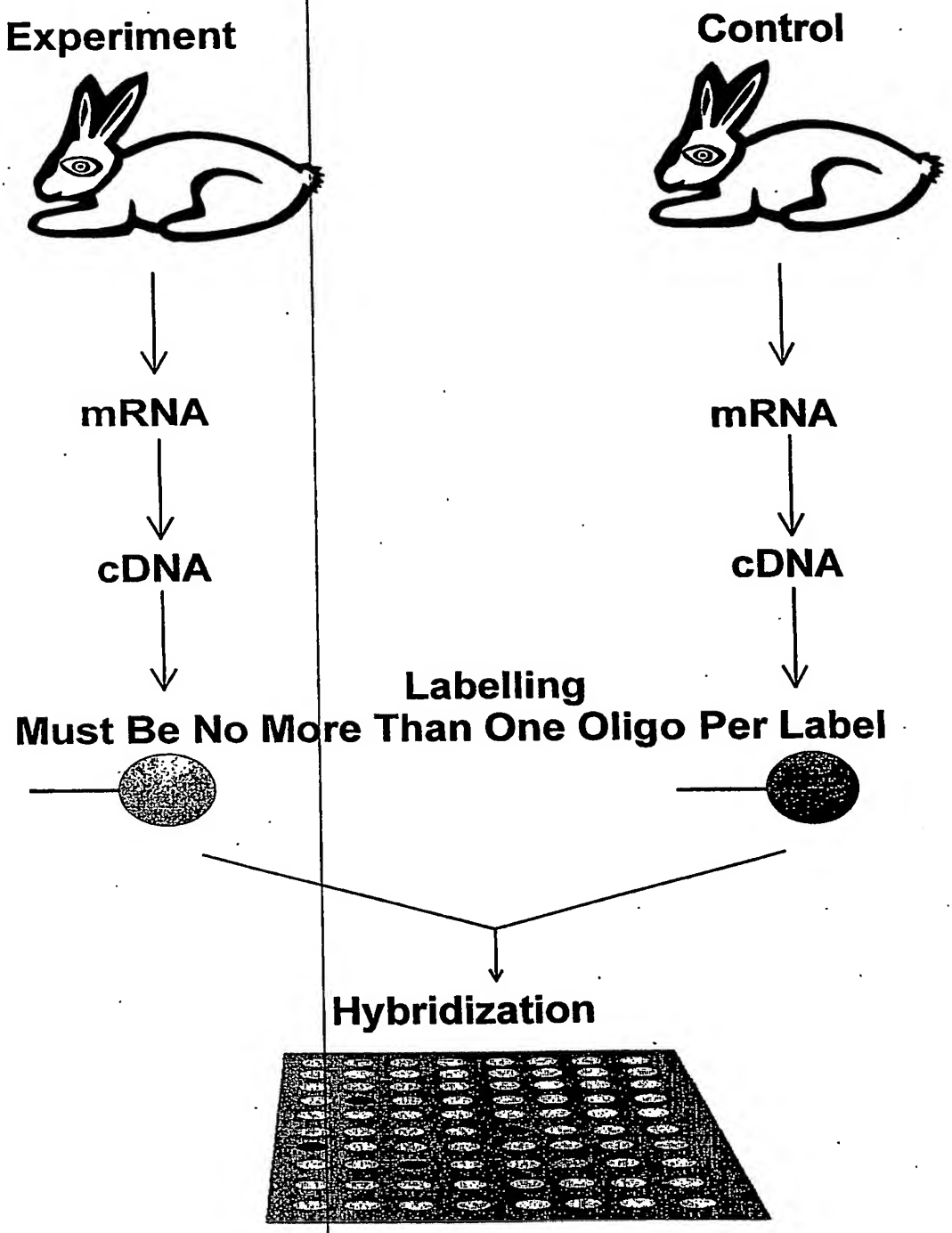
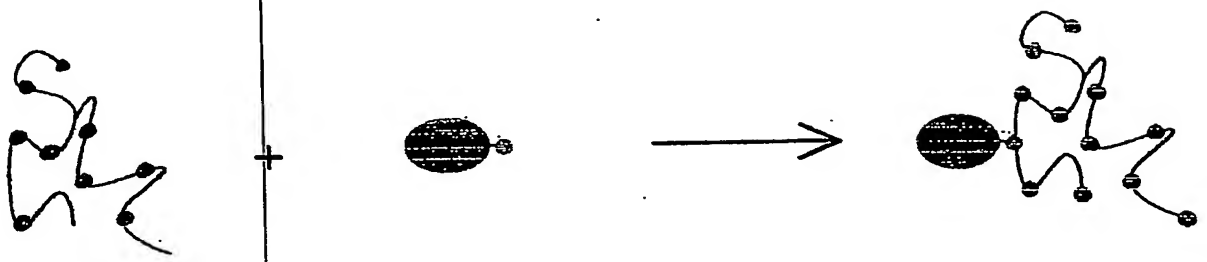

Experiment
Control
mRNA
mRNA
cDNA
cDNA
Labelling
Must Be No More Than One Oligo Per Label
Hybridization

Figure 9

A) Controlled Substitution



B) Self Assembly

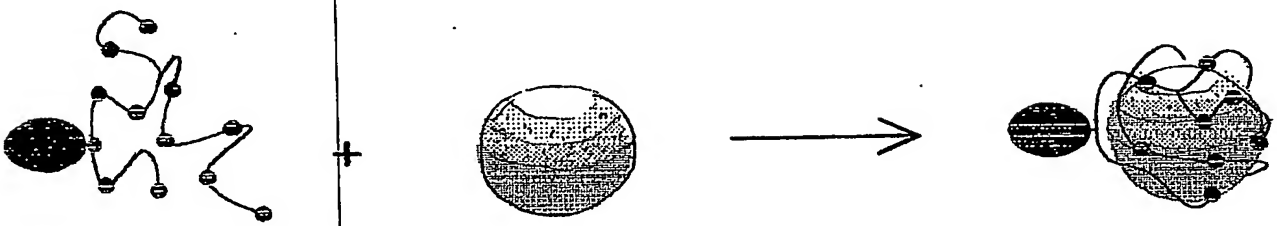
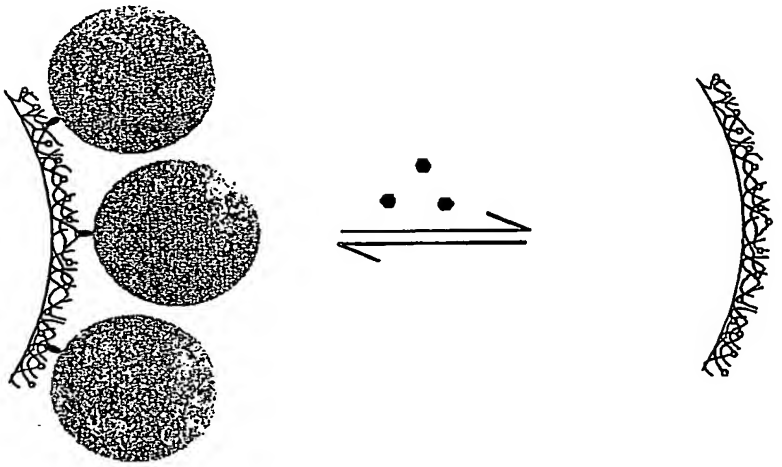


Figure 10

A) Large particles - narrow dynamic range



B) Small particles - broad dynamic range

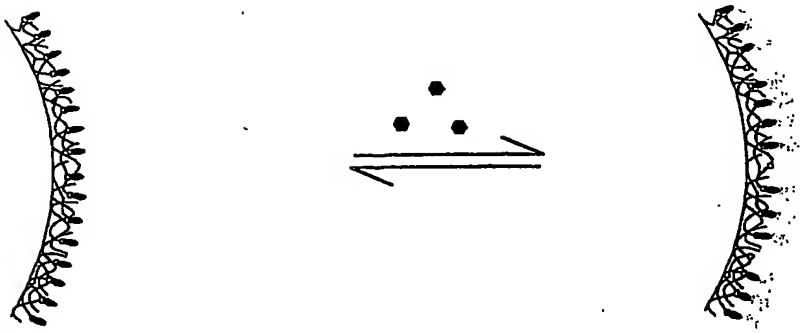
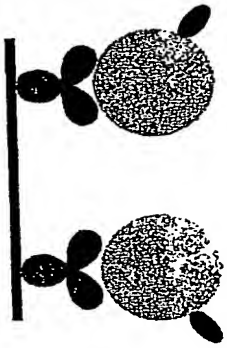


Figure 11

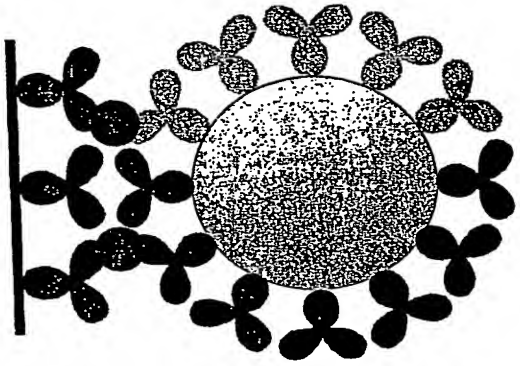
A) Binding reactions of large particles hindered by the particles themselves



B) Small biomolecule-sized partilces are more effctive

Figure 12

A) Large particles - high ambiguity



B) Small biomolecule-sized particles - low ambiguity

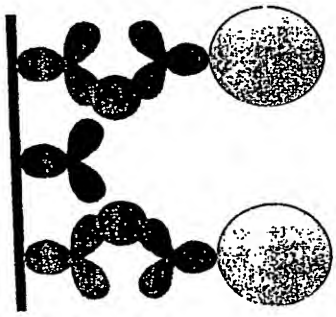


Figure 13

Number Of Mercapto Groups (343 nm) On Adding DTT

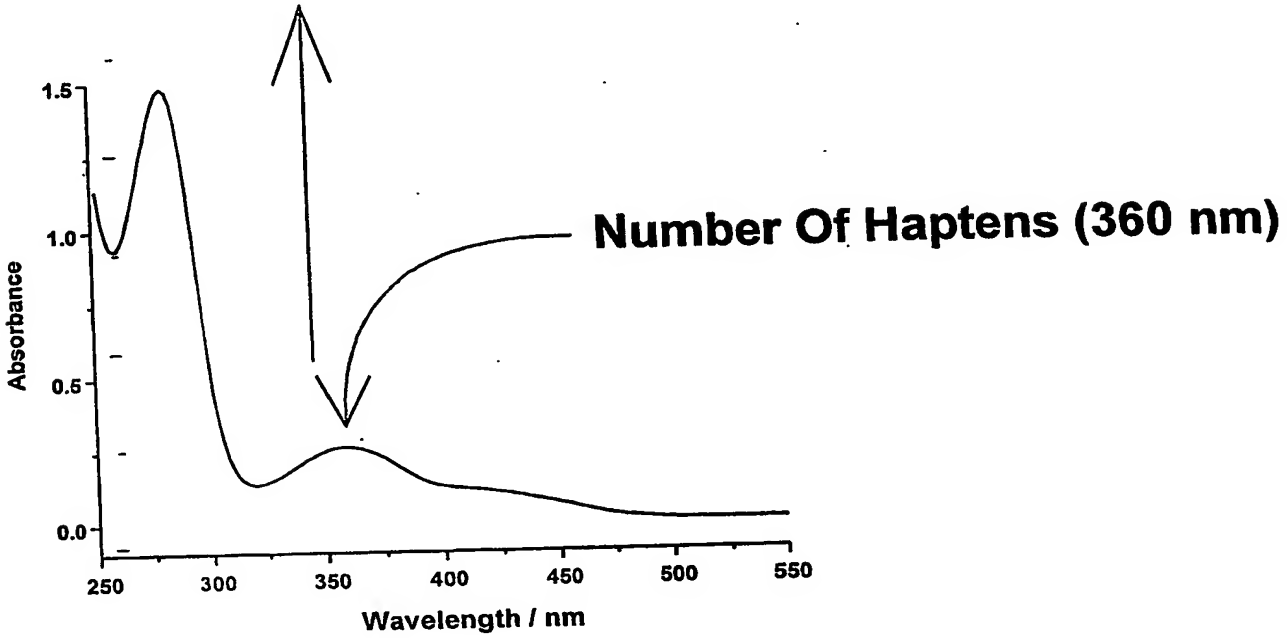
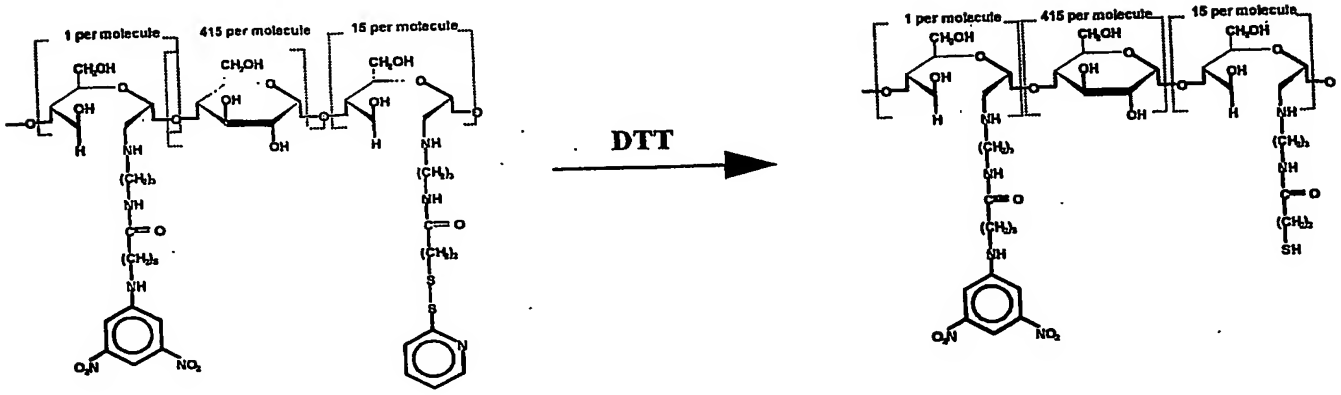


Figure 14



Haptenylated Pyrdyldithiodextran (A Protected Mercaptodextran)

Haptenylated Mercaptodextran

Figure 15

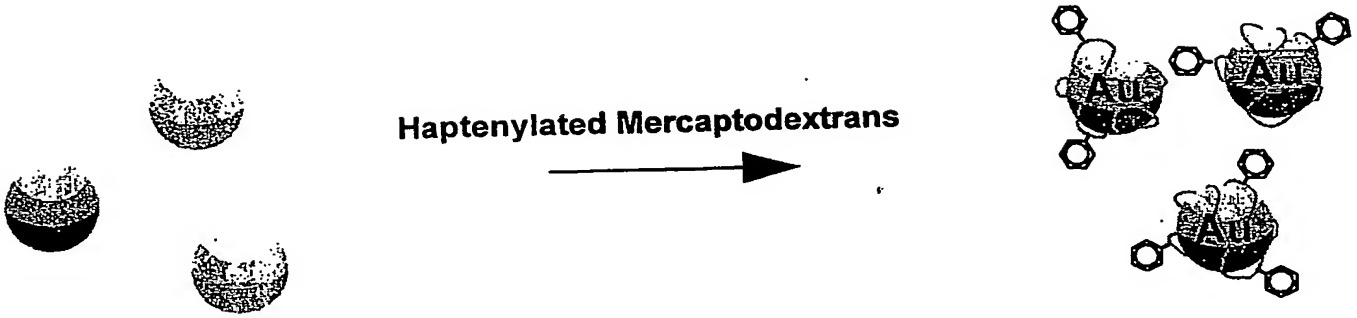


Figure 16

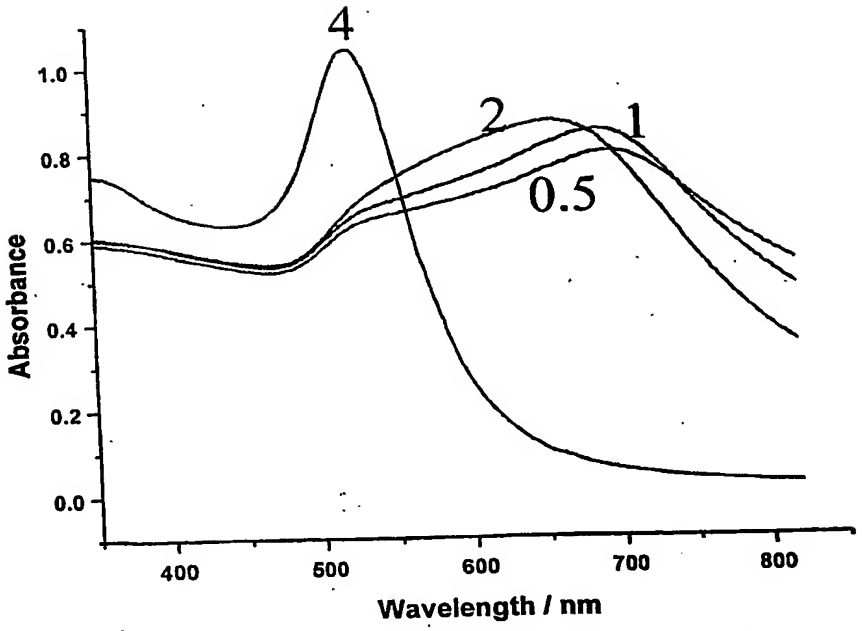


Figure 17

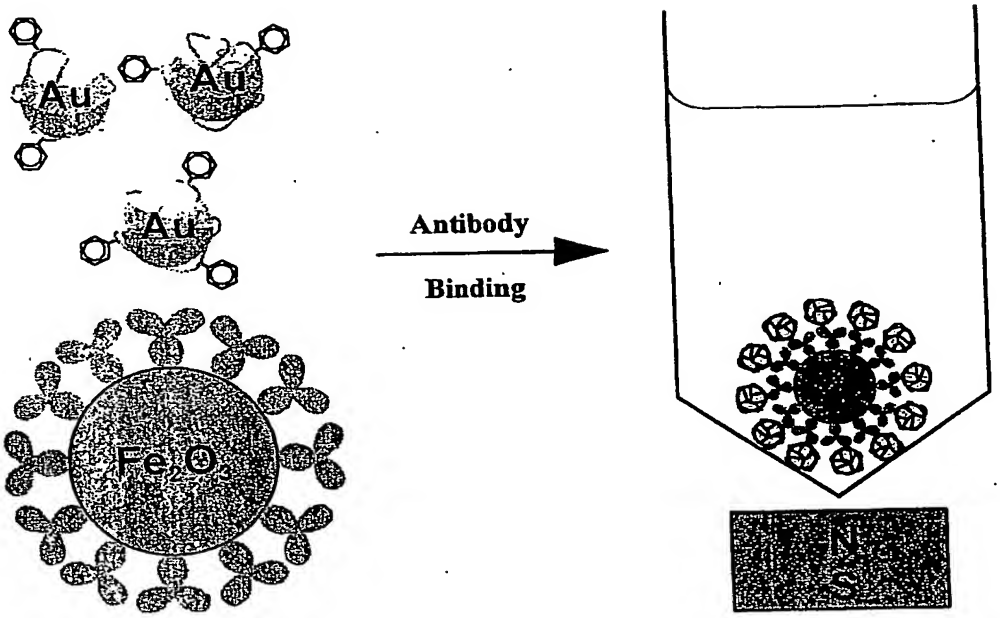


Figure 18

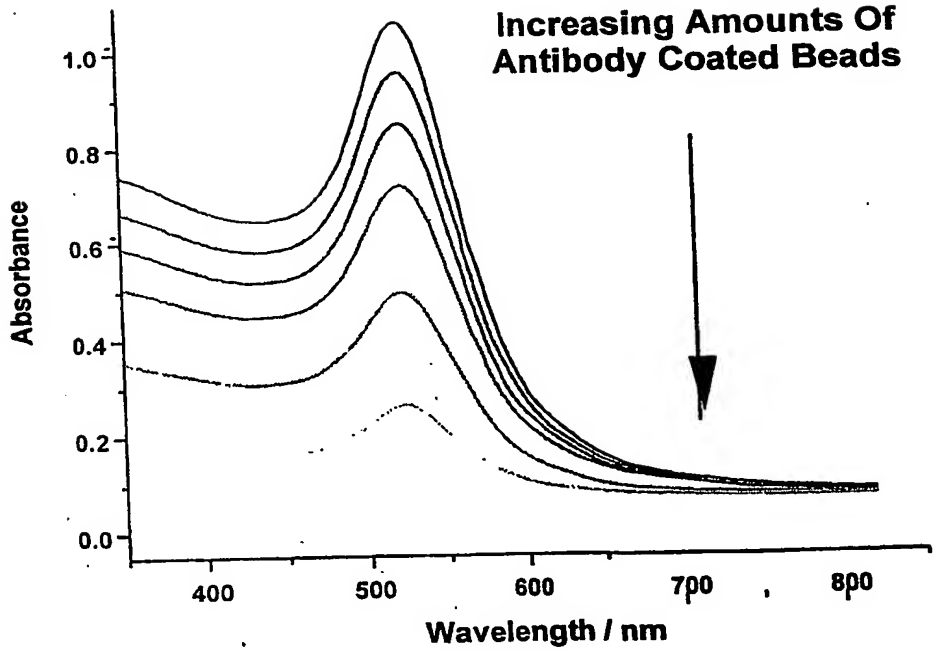
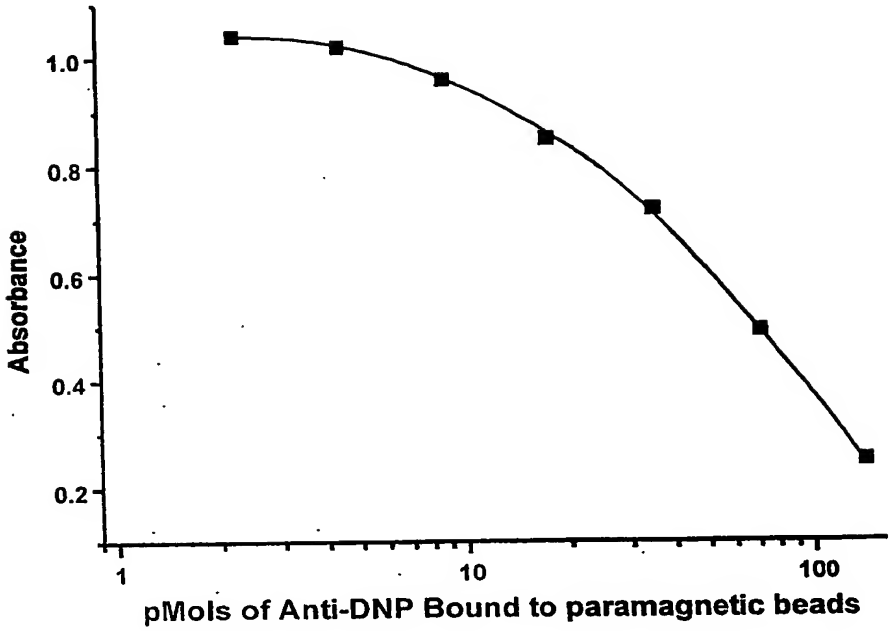


Figure 19


1.0
0.8
0.6
0.4
0.2
Absorbance
1
10
100
pMols of Anti-DNP Bound to paramagnetic beads